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PRINCIPAL INVESTIGATOR: Stanley Zucker, M.D.

CONTRACTING ORGANIZATION: New York State University
at Stonybrook
Stonybrook, New York 11794

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Stanley Zuber 12/20/96
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INTRODUCTION

Tumor Collagenase Stimulating Factor (TCSF) which we have recently renamed Extracellular Matrix Metalloproteinase Inducer (EMMPRIN), based on its stimulatory effect on gelatinase A and stromelysin-1 as well as collagenase production (Kataoka, et al., 1993), is a glycoprotein identified on the plasma membrane of cancer cells which induces fibroblasts to produce Matrix Metalloproteinases (MMPs) (Biswas, et al., 1995). Our hypothesis is that breast cancer cell EMMPRIN induces peri-tumoral fibroblasts to produce the MMPs (collagenase, gelatinase A, and stromelysin-1) required for cancer invasion and metastasis.

Considerable evidence has been presented to support the concept that cancer cells express a signaling mechanism which controls fibroblast production of MMPs (Biswas, et al., 1995). EMMPRIN (TCSF), was purified from the plasma membranes of cancer cells and identified as a 58 kDa glycoprotein which induces fibroblasts to produce interstitial collagenase (MMP-1), thereby facilitating the invasive process (Biswas, et al., 1995, Ellis, et al., 1989, Nabeshima, et al., 1991). EMMPRIN had no mitogenic activity and therefore differs from most well

characterized cytokines, such as IL-1, TNF, and TGF- β . Monoclonal antibodies raised against EMMPRIN from human lung carcinoma cells were used to purify and characterize this membrane glycoprotein (Ellis, et al., 1989). The E11F4 monoclonal antibody also inhibited the biological activity of EMMPRIN, thereby proving that EMMPRIN was the effector molecule. Following development of specific monoclonal antibodies, EMMPRIN was identified by immunohistochemistry in the cell membranes of malignant epithelial cells from tumor specimens of human lung cancer and bladder cancer (Muraoka, et al., 1993). Recent reports from other laboratories have confirmed the stimulatory effect of human breast and bladder cancer cells on production of MMPs by host fibroblasts (Ito, et al., 1995, Muraoka, et al., 1993). Cancer cell membranes and soluble factors exerted different effects on MMP and TIMP production.

The hypothesis that cancer cells produce a factor that stimulates normal cells to synthesize MMPs is supported by recent in situ hybridization studies of human breast cancer tissue which revealed an apparent paradox; cDNA for matrix metalloproteinases (stromelysin-3 and gelatinase A) has been identified in fibroblasts surrounding the tumor rather than in the tumor cells themselves (Basset, et al., 1990, Pyke, et al., 1993). Interpretation of this data has led to the suggestion that normal host fibroblasts produce much of the MMPs that the cancer cells utilizes during invasion. Using immunohistochemistry, we have identified the selective localization of EMMPRIN on the surface of malignant cells in human breast cancer tissue, further suggesting that this factor may provide the missing link to explain the observation that peritumoral fibroblasts are the major producers of MMPs.

Based on the potential importance of EMMPRIN in regulation of MMP activity during tumor cell invasion, we have studied EMMPRIN at the molecular and physiologic level. Following determination of the nucleotide sequence of the cDNA for human EMMPRIN (Biswas, et al., 1995) it was recognized that EMMPRIN is homologous to proteins of the Ig superfamily (Kásinrerker, et al., 1992, Seulberger, et al., 1992) which have been identified in arthritis and embryonic epithelial/stromal interactions; the function of these proteins was not explored in these latter studies, but a function similar to EMMPRIN would be appropriate in these situations.

BODY OF ANNUAL REPORT

Experimental Results (the original timetable for these tasks is listed in parentheses)

Task (1). Identify the cellular localization of EMMPRIN (TCSF) and MMPs in human breast cancer tissue.

(1a) Obtain tissue samples from patients with various forms of breast cancer (24 months):

Breast tissue samples have been obtained from 57 women with various forms of breast cancer and women with benign breast disease. Our original goal was to obtain 100 tissue specimens. Based on the limitations in distinguishing EMMPRIN content between malignant versus benign breast cancer using an immunohistochemical technique (see Section 1b below), additional breast tissue specimens have not been sought. Additional tissue specimens will not be collected until an ELISA is developed to more precisely quantitate EMMPRIN in tissues.

(1b) Immunolocalization of EMMPRIN and MMPs in human breast cancer tissue using specific antibodies to EMMPRIN to determine epithelial:mesenchymal contributions (36 months):

Preliminary results were discussed in the 1995 Annual Report. Using immunohistochemical techniques, we have characterized the cellular localization of EMMPRIN as compared to the localization of gelatinase A in breast cancer. Additional experimental studies have been performed on tumor sections obtained from a total of 28 women who underwent total mastectomy or lumpectomy for breast cancer. In all cases of invasive ductal cancer, antibodies to EMMPRIN reacted strongly with invasive cancer cells with intense staining of the plasma membrane and less intense staining of cytoplasm (Figure 1). In comparison with cancer cells, normal ducts within the tumor specimen demonstrated similar staining with anti-EMMPRIIN antibody. Minimal staining was present in fibroblasts and adipose tissue in the tumor stroma. EMMPRIN immunostaining was intense in both early and advanced stages of invasive breast cancer. Immunostaining of in situ breast carcinomas resulted in epithelial staining as intense as invasive breast cancer. Moderately intense EMMPRIN staining of breast ducts and acini was noted in breast tissue obtained from biopsies of patients with benign breast disease. Quantitative distinction between the intensity of EMMPRIN staining in benign and malignant tissues by further dilution of the anti-EMMPRIIN antibody (1:40, 1:80, 1:160) did not reveal a distinctive difference between EMMPRIN content in benign versus malignant breast tissue.

These results have identified limitations in quantifying EMMPRIN antigen concentrations in human tissue using immunohistochemistry. The presence of considerable EMMPRIN staining in normal breast ducts and acini is not unexpected (see data below on keratinocytes). These data suggest that EMMPRIN may have a function in embryonic development or maintenance of normal breast tissue, as well as the malignant process. We propose that in physiologic processes, the presence of an intact basement membrane separating the normal/benign epithelium from underlying stromal fibroblasts limits access of epithelial cell EMMPRIN for induction of MMP production by stromal fibroblasts through a cell-cell contact related mechanism. In contrast, in carcinomas the epithelial basement membrane is fragmented, thereby permitting epithelial cancer cells to migrate into the stroma, make direct cell contact with fibroblasts, and stimulate fibroblast synthesis of MMPs. The enhanced production of MMPs by peritumoral fibroblasts then leads to degradation of the stroma (including the basement membrane), thereby enhancing the invasive/metastatic process of cancer cells.

(1c) Develop an ELISA for EMMPRIN (TCSF) for use in quantifying antigen in breast tissue (6 months):

Production of monoclonal and polyclonal antibodies to recombinant EMMPRIN for use in ELISAs. We have now immunized a total of 6 BALB/c mice by the intraperitoneal injection of 250 ug of recombinant EMMPRIN (purified from CHO cell homogenates transfected with EMMPRIN) emulsified in RIBI immunoadjuvant (RIBI Immunochemical Systems, Inc.) at

four week intervals X 4-6. High serum titers of antibodies against the immunogen were achieved in each case. Spleen-myeloma cell fusion, growth of hybridomas, and purification of immunoglobulin was accomplished in 1995. The problem with the initial 13 mouse myeloma clones developed was that all of the antibodies were of the IgM type; these IgM antibodies reacted nonspecifically with other proteins as demonstrated by Western blotting. Modification of the screening procedure by employing goat anti-mouse IgG antibodies rather than combined anti-mouse IgG, IgM, and IgA as the detecting reagent, did not ameliorate the problem in the next two clones isolated.

In an attempt to circumvent this problem, we are currently using EMMPRIN purified from human lung cancer cells (LX-1) rather than recombinant EMMPRIN as the immunogen. EMMPRIN antigen injections (50 ug mixed with RIBI immunoadjuvant) in mice are being performed on a 2 week rather than a 4 week schedule and mice are sacrificed after 6 weeks, rather than 4 months as performed in 1995. Antibody titers to EMMPRIN in mouse serum have been higher using the native antigen rather than recombinant antigen. Spleen myeloma cells fusions were performed in late November, 1996 and antibody producing clones are currently being screened for IgG producing clones. Results should be available by February, 1997.

Polyclonal antibodies to EMMPRIN were produced in a rabbit following injection of recombinant human EMMPRIN. Although the rabbit serum titer against human EMMPRIN was significantly increased after 3 injections of antigen, the antibody titer did not increase sufficiently for use of this antibody in a sandwich type immunoassay (ELISA). The rabbit antibody was however specific for EMMPRIN as tested by Western blotting at low antibody dilution.

The development of an ELISA for EMMPRIN has been delayed due to the limited affinity and specificity of EMMPRIN antibodies produced to date. Further efforts to produce better antibodies are underway.

(1d) Quantify the EMMPRIN, gelatinase A, gelatinase B, and stromelysin-1 content of fresh tissue samples obtained from patients with breast cancer (36 months):

The quantification of EMMPRIN content of breast cancer tissue will await the development of an ELISA for EMMPRIN antigen quantification (see description above). The ELISA for gelatinase A, gelatinase B, and stromelysin-1 are currently in place in the Zucker lab, but have not been used on the collected breast tissue samples since it is desirable to examine all of these antigens in a relatively short period of time to avoid problems of antigen decay in stored specimens. Multiple freeze-thaw cycles are to be avoided because of protein denaturation.

(1e) Identify mRNA for EMMPRIN (TCSF) in breast cancer tissue using in situ hybridization (48 months):

This task has been completed ahead of schedule. To characterize and distinguish the cells producing EMMPRIN and gelatinase A in breast cancer, we have employed in situ hybridization using radiolabeled RNA probes for EMMPRIN and gelatinase A (Polette, et al., 1996). Surgical specimens were obtained from 22 women with breast cancer and from 7 women with benign breast disease (fibrocystic disease and fibroadenoma). The slides were treated with proteinase K to remove basic proteins and hybridized with ³⁵S-CTP and -UTP-labeled antisense and sense RNA transcripts for EMMPRIN and gelatinase A. Following stringent washes and autoradiography, slides were counterstained and mRNA was scored by visual assessment of grain counts. The results of these studies was that EMMPRIN mRNA was detected by in situ hybridization in all carcinomas in both non invasive and invasive cancer cells and in pre malignant areas such as atypical hyperplasia of the breast (Figure 2). EMMPRIN mRNA and gelatinase A mRNA were both visualized in the same areas in serial sections in breast cancer, but were expressed by different cells with tumor cells expressing EMMPRIN mRNA and fibroblasts expressing gelatinase A mRNA (Figure 2). There was no correlation between EMMPRIN mRNA and the tumor size, grade of the tumors, the number of lymph node metastases, and the hormonal receptor status of the tumors. Normal mammary

glands mixed with cancer cells or adjacent to cancer areas showed no EMMPRIN hybridization grains. Stromal cells did not express any EMMPRIN hybridization grains. These histologic results were confirmed by Northern blot analysis of tissue extracts which demonstrated higher expression of EMMPRIN mRNAs in breast cancers than in benign and normal breast tissue (Figure 3). The Northern blot data are consistent with the presence of far greater numbers of epithelial cells in malignant tissues than in normal breast tissue. The discrepancy noted between normal breast ducts staining positively for EMMPRIN by immunohistochemistry but negatively by in situ hybridization suggests differences in sensitivity of these techniques for EMMPRIN detection or differences in the rates of EMMPRIN turnover in normal versus malignant tissues. These observations support the hypothesis that EMMPRIN is an important factor in tumor progression in vivo in breast cancer by stimulating fibroblast production of matrix metalloproteinases. A manuscript presenting this data is in press (Polette, et al., 1996). Future studies will be directed toward explaining the lack of correlation between data from in situ hybridization and immunohistochemistry for EMMPRIN.

Task (2). Identify important structural:functional relationships in the EMMPRIN (TCSF) molecule.

(2a) Determine whether post-translational processing is required for biological activity (12 months):

In 1995, we expressed human EMMPRIN as a fusion protein with Glutathione-S-transferase in the prokaryotic pGEX vector. The fusion protein was purified from lysed bacteria under nondenaturing conditions. A single protein band was obtained of ~28 kDa in SDS PAGE and Western blotting. The recombinant non-glycosylated protein encoded by cDNA for EMMPRIN in *E. coli* was not active in stimulating human fibroblast metalloproteinase synthesis. To circumvent this problem, we stably transfected mammalian cells (CHO) with EMMPRIN cDNA in order to allow posttranslational processing to take place, and then selected cells producing high levels of fully processed EMMPRIN. The EMMPRIN produced by these cells was localized to the cell surface and was of similar molecular weight to native EMMPRIN from tumor cells i.e. ~58 kDa. We immunopurified the recombinant EMMPRIN after extraction from CHO cell membranes using monoclonal antibodies raised against native tumor cell EMMPRIN (Figure 4). When added to human fibroblasts in culture, the purified recombinant EMMPRIN was found to be active in stimulating production (2-5 fold) of fibroblast interstitial collagenase, gelatinase A, and stromelysin-1 (Table 1), but not TIMP-1 (Guo, et al., 1997). Since non-glycosylated and partially glycosylated recombinant EMMPRIN were unable to stimulate MMP production, we conclude that post-translational processing is required for EMMPRIN activity. This effect would result in an imbalanced production of active MMPs and the potential, in vivo, of enhanced tumor cell invasion as a result of stromal degradation.

We have also shown that EMMPRIN purified from stably transfected CHO cells stimulates production of stromelysin-1 and gelatinase A (demonstrated by ELISA) by human umbilical vein endothelial cells (manuscript in preparation). This important data suggests that endothelial cells respond to the MMP-inducing effects of EMMPRIN in a similar manner to fibroblasts. We propose that in vivo, direct contact between circulating tumor cells and endothelial cells lining blood vessels at organs distant from the primary tumor may facilitate tumor cell penetration of the subendothelial basement membrane during metastasis. In this scenario, tumor cells induce endothelial cells to secrete MMPs that subsequently facilitate basement membrane degradation.

(2b) Alter EMMPRIN (TCSF) by deletional mutation and site directed mutagenesis of cDNA and then analyze mutant proteins to determine the minimum amino acid sequences necessary for functional activity (36 months):

Since we know that post-translational processing is essential for EMMPRIN activity, we have begun to prepare mutant EMMPRIN protein with altered glycosylation sites. This work has only recently begun, and will continue through 1997. In 1997, we also plan to express

polypeptides that lack specific regions of either domain I or domain II of the extracellular region of EMMPRIN in pGEX as described in the original grant application.

(2c) Design peptide antagonists and produce anti-functional monoclonal antibodies to further characterize the structure: functional relationship of the EMMPRIN molecule (48 months):

This task will be initiated after task 2b is completed.

Task 3. Explore the role of EMMPRIN (TCSF) in cancer dissemination using experimental models.

(3a) Compare the effect of transfecting breast cancer cells with cDNA for native versus mutant EMMPRIN in regards to altering cancer invasion and metastasis in an experimental model (48 months):

In our initial experiments, we have transfected the open reading frame for EMMPRIN cDNA into human breast cancer cell lines (MDA-MB-436 and MCF-7). Transfected cell lines were then injected into the mammary fat pad of 6-8 week old nude female mice at a cell concentration of 1×10^6 per animal. Palpable tumors (1-4 cm in diameter) were detected after 3 months in 2/4 mice injected with EMMPRIN cDNA- transfected MDA-MB-436 cells and in 3/4 mice with mock transfected MDA-MB-436 cells. Metastases were noted in the local lymph nodes of 1/4 mock transfected cells, but not in EMMPRIN-transfected cells. Tumor size between EMMPRIN and mock transfected tumor injected animals were comparable. Histology of the breast cancers was as anticipated indicating that the morphologic phenotype had not changed.

Based on this preliminary result, the experimental protocol will be modified to enhance the percent of positive primary tumors developing and to increase the possibility of distant metastasis. Modifications may include: 1) obtaining a fresh batch of cancer cells for transfection, 2) injection of larger numbers of cancer cells, 3) modifying the procedure for construction of vector.

To ascertain that transfected tumor cells are expressing EMMPRIN protein in high concentration, we will take advantage of the recent observation that the green fluorescent protein (GFP) of the jelly fish *Aequoria victoria* retains its fluorescent properties when recombinantly expressed in eukaryotic cells (Rizzuto, et al., 1995). This 29 kDa protein can then be used as a powerful marker for gene expression in vivo (Hampton, et al., 1996). Many studies have used this reporter gene for protein localization in cells. Recently, we have cloned EMMPRIN cDNA along with the GFP reporter cDNA as a fusion gene into pcDNA3 expression vector. We identified the GFP-EMMPRIN fusion protein in the plasma membrane of transfected COS-1 cells using fluorescent microscopy, thus indicating that GFP is transported to the plasma membrane along with the EMMPRIN protein. It is unknown whether this fusion protein expresses MMP stimulating effects. Our future experimental strategy is to: 1) establish stably transfected tumor cells for the above plasmid and a second plasmid in which GFP and EMMPRIN are controlled individually by separate CMV promoters using selection for resistant colonies to G418, 2) identify EMMPRIN expression by fluorescent microscopy and immunoblotting of transfected cell lysates, 3) use FACS to select populations with high GFP expression and low GFP expression, presumably reflecting high and low levels of EMMPRIN, respectively, 4) inject nude mice with these cell lines and examine for tumorigenicity and metastasis.

(3b) Analyze human peritumoral fibroblast response to EMMPRIN (TCSF) in vitro (36 months):

These experiments will be initiated in 1997 as originally planned.

Additional tasks not listed in the original grant application

Task 4. Human Keratinocyte EMMPRIN.

Initial studies showed that EMMPRIN is not present in significant amounts on many types of normal adult human cells (Ellis, et al., 1989, Muraoka, et al., 1993). However, after obtaining cDNAs for EMMPRIN, it became apparent that EMMPRIN is identical to human basigin and M6 antigen, (Kasinrerk, et al., 1992, Seulberger, et al., 1992) and is expressed in some physiologically active epithelia during embryonic development, as well as tumor cells. The distribution of EMMPRIN/basigin/M6 antigen appears to be restricted to certain activated leukocytes and to some physiologically active epithelia, as well as to tumor cells. Since keratinocytes have previously been shown to stimulate MMP production by fibroblasts (Johnson-Wint and Bauer, 1985) and since epithelial-dermal interactions are important in preserving and repairing skin structures, we sought evidence for the presence of EMMPRIN in keratinocytes. We found that human keratinocytes express EMMPRIN at their cell surface in vivo and in vitro and synthesize EMMPRIN in culture, albeit at a lower level than tumor cells. On characterization of EMMPRIN cDNA obtained from a keratinocyte cDNA library, we found that the deduced amino acid sequence was identical to that of tumor cell EMMPRIN (DeCastro, et al., 1996). These cDNAs share a common region of 1459 nucleotide residues that differ in only 7 of these residues, only two of which are in the open reading frame and which result in no differences in the amino acid sequence of EMMPRIN. The significance, if any, of the polymorphism in the cDNA sequence of the two clones is unclear. We conclude that human keratinocytes produce EMMPRIN; however, we have not yet ascertained whether it is active nor whether it is involved in matrix turnover at the epidermis-dermal interface. A role for EMMPRIN at epithelial dermal junctions in tissue repair during wound healing seems highly plausible. Taken together, these data suggest that the function of EMMPRIN is under strict regulation in normal tissues, but this control mechanism may go awry in cancer.

Task 5. Human EMMPRIN gene.

We have commenced characterization of the EMMPRIN gene. We have shown that a single gene for EMMPRIN is situated on chromosome 19 (19p13-3). The gene is ~25 kilobase size. We have characterized all of the exon/intron boundaries over the translated region (this region is ~5 kbase in length). We have identified the transcription initiation site and sequenced ~1 kbase 5' of this site. We are currently examining this region for known motifs characteristic of promoter regions (manuscript in preparation).

CONCLUSIONS

EMMPRIN (TCSF) is a plasma membrane glycoprotein that is present on the surface of breast cancer cells, and is responsible, in part, for the elevated levels of MMPs in peritumoral fibroblasts and endothelial cells. EMMPRIN requires post-translational processing (glycosylation) for its ability to stimulate production of MMPs in target fibroblasts. Transfection of EMMPRIN cDNA into CHO cells resulted in the production of a glycosylated functional protein of similar molecular weight to native EMMPRIN (58 kDa) that was localized to the plasma membrane. EMMPRIN mRNA is expressed in benign and malignant human mammary ducts and acini to a much greater degree than in normal breast ducts. Immunohistochemical approaches, however, have indicated that EMMPRIN is also present in normal breast ducts and some other epithelial structures (i.e. keratinocytes). The discrepancy between in situ hybridization and immunohistochemistry needs to be explained by development of an immunoassay for EMMPRIN in tissues. In addition, we have shown that a single gene for EMMPRIN (~25 kilobase size) is situated on chromosome 19 (19p13-3) and have characterized all of the exon/intron boundaries over the translated region (this region is ~5 kb in length). From a therapeutic point of view, inhibition of EMMPRIN may provide a potential mechanism to alter the invasive process in breast cancer.

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Figure 1. Immunohistochemistry for EMMPRIN antigen in human breast tissue. Breast tissue from surgical specimens was stained with mouse monoclonal anti-human EMMPRIN antibodies. An alkaline phosphatase labeled streptavidin-biotin method (LSAB) was used to label tissue sections as per manufacturer's instructions. After the primary antibody reacted with the antigen, a biotinylated link immunoglobulin was added to the tissue. Then an alkaline phosphatase conjugated streptavidin label was added to the tissue. Rinses were used between each step to remove excess antigen. Finally, the Fast Red/Substrate solution was allowed to react with the tissue (reagents produce by Biogenex Labs). The alkaline phosphatase reaction yields an intense red color at the localized antigen site. Panel A represents the negative control (non-immune mouse IgG) for panel B (breast intraductal carcinoma in situ). Panel C contains normal breast ductules (benign breast tissue specimen). Panel D contains invasive ductal breast cancer. The intensity of positive immunostaining (red color) is comparable between the normal ductules, carcinoma in situ, and invasive breast cancer. Fibroblasts and extracellular matrix are minimally stained in normal or malignant tissues.

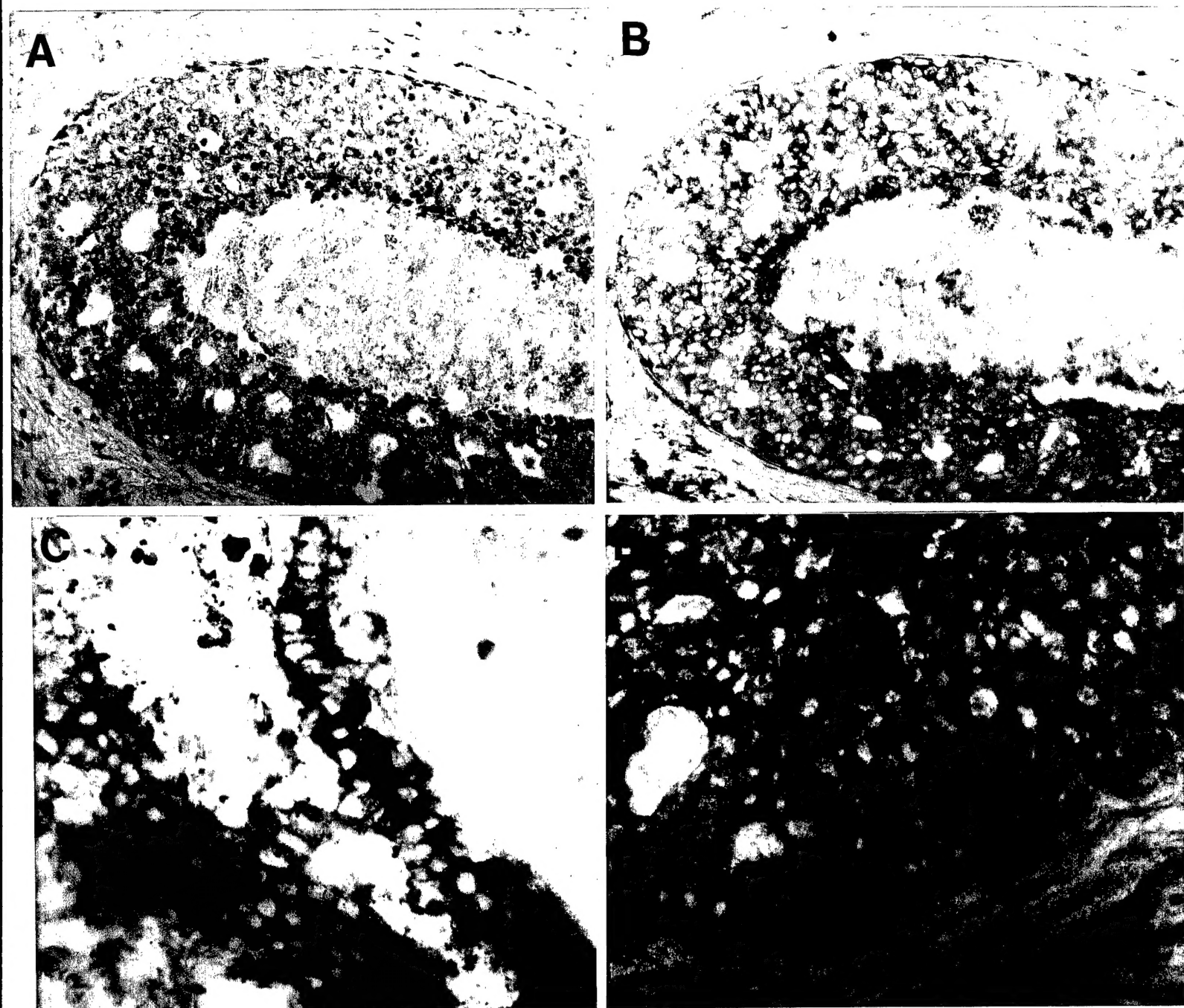


Figure 2. Identification of mRNA for EMMPRIN (TCSF) in breast cancer tissue using in situ hybridization. Panel A represents normal breast lobules; no hybridization for EMMPRIN is noted. Panel B represents EMMPRIN hybridization in invasive breast cancer. Panel C represents EMMPRIN in well differentiated breast cancer (T), but no hybridization in normal adjacent tissue. Panel D represents gelatinase A hybridization in fibroblasts surrounding breast cancer tissue, but minimal gelatinase A in tumor cells.

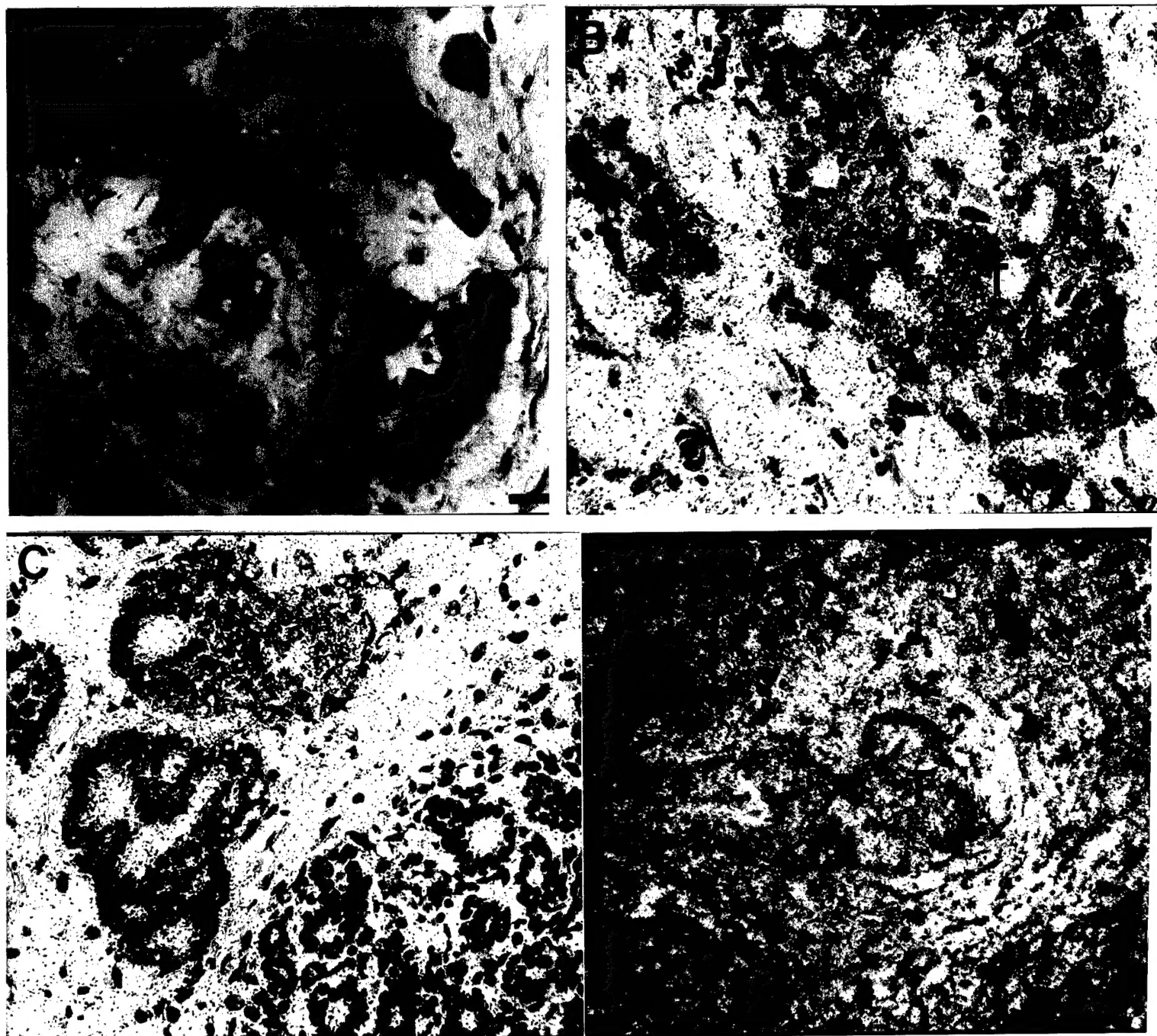


Figure 3. Northern blot analysis of EMMPRIN in breast samples. Rows A and B contain mRNA from 22 breast cancer specimens. Row C contains mRNA from benign breast lesions. As noted, the amount of EMMPRIN mRNA (1.7 kb) was increased in all breast cancer specimens as compared to benign breast lesions.

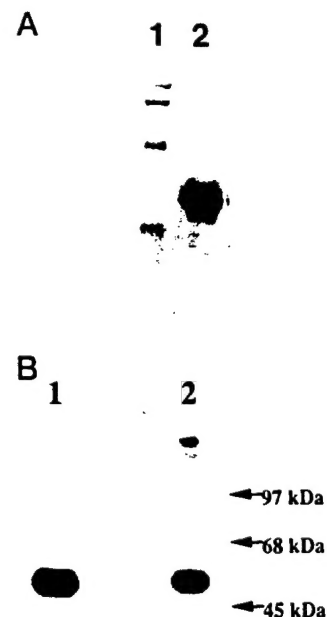
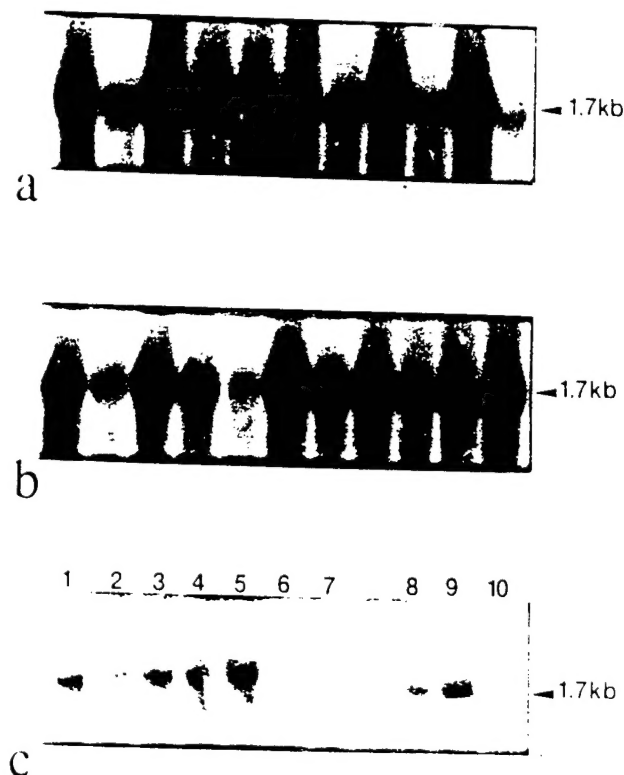


FIG. 4. SDS-PAGE and Western blotting of purified recombinant EMMPRIN. A, silver-stained SDS-PAGE gel of EMMPRIN purified from CHO cells transfected with EMMPRIN cDNA. EMMPRIN was purified from cell membranes as described under "Experimental Procedures," dissolved in SDS sample buffer containing 0.1 M dithiothreitol, heated at 95 °C for 10 min, and subjected to 10% SDS-PAGE; the gel was deliberately overloaded to reveal potential contaminants. Lane 1, molecular mass standards (45, 66, 97, and 116 kDa); lane 2, purified recombinant EMMPRIN. B, Western blot of recombinant EMMPRIN purified from CHO cells transfected with EMMPRIN cDNA (lane 1) and of native EMMPRIN purified from LX-1 cells (lane 2). A 10% SDS-PAGE gel was electroblotted to a nitrocellulose membrane followed by blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20. The blot was incubated with E11F4 hybridoma supernatant (13) for 1 h at room temperature and then with horseradish peroxidase-conjugated anti-mouse IgG. The EMMPRIN protein bands were detected with ECL Western blotting detection reagents (Amersham Corp.). In both cases the anti-EMMPRIN antibody recognized a protein with a molecular mass of ~58 kDa. Some immunoreactive, aggregated protein was also present in LX-1 cells, as previously noted (14).

TABLE I
Stimulation of MMP production by recombinant EMMPRIN
Recombinant EMMPRIN was purified from membranes of transfected CHO cells and, in two separate experiments, added at 100 µg/ml to cultures of human fibroblasts. Cultures were also incubated with TPA (0.1 µg/ml) or with no added reagent. After incubation, aliquots of culture medium were used for ELISA of MMP-1, MMP-2, and MMP-3. Amounts of MMP are expressed as µg/ml ± S.E.

Agent added	MMP-1	MMP-2	MMP-3
Experiment 1			
None	0.03 ± 0.00	1.40 ± 0.01	0.21 ± 0.01
rEMMPRIN	0.33 ± 0.02 ^a	2.12 ± 0.13 ^a	0.93 ± 0.13 ^a
TPA	0.32 ± 0.02 ^a	2.33 ± 0.29 ^a	0.42 ± 0.02 ^a
Experiment 2			
None	0.03 ± 0.00	0.13 ± 0.01	0.35 ± 0.04
rEMMPRIN	0.17 ± 0.02 ^a	2.10 ± 0.37 ^a	0.63 ± 0.03 ^a
TPA	0.25 ± 0.02 ^a	0.46 ± 0.06 ^a	0.56 ± 0.07 ^a

^a Significantly greater than control (none added), *p* < 0.05.